HLA-DR15-derived self-peptides are involved in increased autologous T cell proliferation in multiple sclerosis

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Abstract: The HLA-DR15 haplotype confers the largest part of the genetic risk to develop multiple sclerosis, a prototypic CD4+ T cell-mediated autoimmune disease. The mechanisms how certain HLA-class II molecules functionally contribute to autoimmune diseases are still poorly understood, but probably involve shaping an autoimmune-prone T cell repertoire during central tolerance in the thymus and subsequently maintaining or even expanding it in the peripheral immune system. Self-peptides that are presented by disease-associated HLA-class II molecules most likely play important roles during both processes. Here, we examined the functional involvement of the HLA-DR15 haplotype in autologous proliferation in multiple sclerosis and the contribution of HLA-DR15 haplotype-derived self-peptides in an in vitro system. We observe increased autologous T cell proliferation in patients with multiple sclerosis in relation to the multiple sclerosis risk-associated HLA-DR15 haplotype. Assuming that the spectrum of self-peptides that is presented by the two HLA-DR15 allelic products is important for sustaining autologous proliferation we performed peptide elution and identification experiments from the multiple sclerosis-associated DR15 molecules and a systematic analysis of a DR15 haplotype-derived self-peptide library. We identify HLA-derived self-peptides as potential mediators of altered autologous proliferation. Our data provide novel insights about perturbed T cell repertoire dynamics and the functional involvement of the major genetic risk factor, the HLA-DR15 haplotype, in multiple sclerosis.

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HLA-DR15-Derived Self-Peptides Are Involved in Increased Autologous T Cell Proliferation in Multiple Sclerosis

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ABSTRACT

The HLA-DR15 haplotype confers the largest part of the genetic risk to develop multiple sclerosis, a prototypic CD4+ T cell-mediated autoimmune disease. The mechanisms how certain HLA-class II molecules functionally contribute to autoimmune diseases are still poorly understood, but probably involve shaping an autoimmune-prone T cell repertoire during central tolerance in the thymus and subsequently maintaining or even expanding it in the peripheral immune system. Self-peptides that are presented by disease-associated HLA-class II molecules most likely play important roles during both processes. Here, we examined the functional involvement of the HLA-DR15 haplotype in autologous proliferation in multiple sclerosis and the contribution of HLA-DR15 haplotype-derived self-peptides in an *in vitro* system. We observe increased autologous T cell proliferation in multiple sclerosis patients in relation to the multiple sclerosis risk-associated HLA-DR15 haplotype. Assuming that the spectrum of self-peptides that is presented by the two HLA-DR15 allelic products is important for sustaining autologous proliferation we performed peptide elution and identification experiments from the multiple sclerosis-associated DR15 molecules and a systematic analysis of a DR15 haplotype-derived self-peptide library. We identify HLA-derived self-peptides as potential mediators of altered autologous proliferation. Our data provide novel insights about perturbed T cell repertoire dynamics and the functional involvement of the major genetic risk factor, the HLA-DR15 haplotype, in multiple sclerosis.

KEY WORDS:

HLA-DR15, Autologous T Cell Proliferation, Multiple Sclerosis, Self-Peptides, Homeostatic Proliferation

ABBREVIATIONS:

APCs = antigen presenting cells; BLS = bare lymphocyte syndrome; CNS = central nervous system; HD = healthy donor; HLA = human leukocyte antigen; IL = interleukin; MHC = major histocompatibility complex; PBMCs = peripheral blood mononuclear cells; TCR = T cell receptor

INTRODUCTION

Multiple sclerosis (MS) is considered a CD4+ T cell-mediated autoimmune disorder of the central nervous system characterized by inflammation, demyelination, glial scarring and axonal/neuronal loss (Sospedra and Martin, 2005). Regarding disease etiology, genome-wide association studies have identified a large number of genetic loci, which, together with multiple environmental factors, confer multiple sclerosis risk (Sawcer *et al.*, 2011). The association between HLA-DR15 (formerly called DR2) and multiple sclerosis was first noticed in 1973 (Jersild *et al.*, 1973), and since then has been among the most reproduced findings in major histocompatibility complex (MHC) genetics (Oksenberg *et al.*, 2008). Furthermore, HLA-DR15 also influences clinical aspects like disease onset and phenotype (Hensiek *et al.*, 2002). However, the mechanisms how the presence of a specific HLA-DR type, of other multiple sclerosis risk alleles and environmental factors translate to disease are largely unknown. Data from animal models and immunological studies in multiple sclerosis patients point at a role of autoreactive CD4+ T cells in multiple sclerosis (Sospedra and Martin, 2005). The most important, albeit indirect finding supporting this notion is the above fact that approximately 10 - 60% of the genetic risk stems from the HLA-DR15 haplotype consisting of two DR alleles, DRB1*15:01 (the heterodimer of DRA1*01:01/DRB1*15:01 is also referred to as DR2b) and DRB5*01:01 (the heterodimer of DRA1*01:01/DRB5*01:01 is also referred to as

DR2a) (Oksenberg *et al.*, 2008). Interestingly, the influence of the environmental risk factors that have been identified for multiple sclerosis thus far, i.e. infection with Epstein-Barr virus, low vitamin D levels, and smoking, is enhanced in DR15+ multiple sclerosis patients (Kakalacheva *et al.*, 2011).

HLA-class II molecules are expressed by antigen-presenting cells (APCs). They present peptides derived from processing of self- or exogenous proteins, and the complexes of self HLA-class II and antigenic peptides serve as recognition structures for CD4+ T cells. In the thymus HLA-class II molecules are involved in shaping the CD4+ T cell receptor (TCR) repertoire through central tolerance mechanisms of positive- and negative selection of T cells (Ashton-Rickardt *et al.*, 1994). Once in the peripheral immune system the T cell repertoire is maintained alive by homeostatic proliferation, and interactions between CD4+ T cells and their TCRs with HLA-class II/self-peptide complexes as well as the cytokines interleukin-7 (IL-7) and IL-15 are the most important stimuli during this process (Sprent and Surh, 2011). Despite the well known role of HLA-class II in TCR repertoire generation and maintenance, our understanding about the functional contribution of HLA-class II molecules to human autoimmune diseases in general and also to multiple sclerosis is, however, still limited.

Regarding possible mechanisms several hypotheses have been developed including preferential presentation of self-peptides with similarities to foreign antigens but also tissue-specific autoantigens (Wucherpfennig and Strominger, 1995), less complete thymic deletion of autoreactive T cells (Klein *et al.*, 2000), and differential HLA-class II expression in certain immune cells and the brain (Prat *et al.*, 2005). Another interesting possibility, which has not been examined thus far in multiple sclerosis, is that distinct structural features of the HLA-DR15 haplotype, i.e. of HLA-DR molecules themselves or the complexes between HLA molecule and self-peptide,

operate via homeostatic proliferation and/or autologous expansion to shape an autoimmunity-prone T cell repertoire (Haegert, 2011). Homeostatic T cell proliferation depends on interactions between TCR and self-peptides presented by HLA molecules and the cytokines IL-7 and IL-15 (Sprent and Surh, 2011). Interestingly, genes coding for components of these and related molecules, i.e. DR15, IL7RA, IL-7, and IL2RA, are associated with multiple sclerosis risk (Sawcer *et al.*, 2011).

Evidence for alterations in CD4+ T cell selection and/or peripheral T cell repertoire maintenance in multiple sclerosis are: myelin-reactive T cells with a proinflammatory phenotype, lack of CD28 expression, and higher antigen avidity are increased in multiple sclerosis patients (Martin *et al.*, 1990; Ota *et al.*, 1990; Pette *et al.*, 1990; Markovic-Plese *et al.*, 2001; Bielekova *et al.*, 2004). Further, reduced thymic output of CD4+ T cells has been observed in multiple sclerosis (Hug *et al.*, 2003; Duszczyszyn *et al.*, 2006; Haegert *et al.*, 2011) as well as reduced numbers and function of CD4+ T regulatory cells (Viglietta *et al.*, 2004). Studies of the T cell repertoire by complementary region determining region 3 spectratyping indicate reduced diversity in multiple sclerosis patients compared to age-matched controls (Laplaud *et al.*, 2004). These observations indicate alterations of the peripheral proliferation and survival/expansion of CD4+ T cells, which might affect antigen avidity and frequency of autoreactive T cells. Hence, alterations in T cell homeostasis could contribute to T cell repertoire perturbations in multiple sclerosis.

Given the prominent role of HLA-DR15 for multiple sclerosis risk, we therefore wanted to investigate its functional involvement in autologous T cell expansion in multiple sclerosis. For this purpose we devised an *in vitro* method to assess autologous proliferation. Different from the previously well examined autologous mixed lymphocyte reaction of human peripheral blood mononuclear cells (PBMCs), which is reduced in multiple sclerosis and other autoimmune diseases (Hafler *et al.*,

1985), we did not expose autologous responder (T lymphocytes) cells to autologous, gamma-irradiated non-T cells as stimulators, but rather seeded unmanipulated PBMCs in a serum-free media. Hereby we addressed first, if autologous T cell proliferation is altered in multiple sclerosis patients in general and if there is a relation to the DR15 haplotype. Next, we dissected the relative role of self-peptides bound to DR2a and DR2b by two approaches, a) elution and identification of self-peptides bound to DR2a or DR2b, and b) based on the fact that HLA-derived-peptides themselves are frequently presented by HLA-class II alleles, we used overlapping peptides spanning the sequences of the two DR- and the DQ molecules of the DR15 haplotype, i.e. the non-polymorphic DRα chain DRA1*01:01, the two DRβ chains DRB1*15:01 and DRB5*01:01, and the tightly linked DQw6 molecule consisting of DQA1*01:02 and DQB1*06:02, as antigens. Our data demonstrate that autologous proliferation is increased in multiple sclerosis patients, which is related to the presence and dose of HLA-DR15 expression, that this phenomenon is observed with both DR alleles expressed in the DR15 haplotype and that HLA-class II-derived self-peptides are likely involved as antigens.

MATERIALS AND METHODS

Patients and healthy donors

Multiple sclerosis patients had clinically definite multiple sclerosis by clinical- and/or McDonald criteria. We examined 69 untreated multiple sclerosis patients, 8 patients with clinically isolated syndrome (CIS), a prestage of relapsing-remitting multiple sclerosis (RR-MS), which often evolves into multiple sclerosis based on MRI- and clinical findings, 37 patients with RR-MS (8 of these in exacerbation), 8 secondary-progressive multiple sclerosis (SP-MS) patients, i.e. the multiple sclerosis form that evolves from RR-MS, and finally 8 primary-progressive multiple sclerosis

(PP-MS) patients. A signed informed consent was obtained from all patients and donors under a protocol that was approved by the Ethics Committee of the Hamburg Board of Physicians (No. 2758). Samples were typed for HLA class II (DRB1*, DRB3*, DRB4*, DRB5*, DQA1* and DQB1*) at the Department of Transfusion Medicine, UKE Hamburg by a reverse SSO (Sequence Specific Oligos) in-house test. In some cases the results were verified by a commercial rSSO test (Dynal RELI-SSO, Invitrogen) or by Atria SBT (Sequence Based Typing, Abbott). HLA-DR15+ indicates the haplotype HLA-DRB1*15:01, -DRB5*01:01, -DQA1*01:02 and –DQB1*06:02. *X indicates another HLA class II haplotype, and *protective indicates HLA-DRB1*14 or –DRB*11 haplotypes (Ramagopalan *et al.*, 2007). PBMCs were freshly isolated from EDTA-containing blood tubes or from buffy coats by Ficoll density gradient centrifugation (PAA, Pasching, Austria). All isolated PBMCs were cryopreserved in media containing 10% dimethyl sulfoxide (DMSO; AppliChem, Darmstadt, Germany) and stored at -180°C.

Proliferative assays

After thawing 2 x 10^5 PBMCs/well (average of 22 wells per donor) were cultured with serum-free AIM-V medium (GIBCO, Invitrogen), containing 2 mM L-glutamine, 50 µg/ml streptomycin sulfate, 10 µg/ml gentamicin sulfate and human albumin, in 96-well U-bottom microtiter plates (Greiner Bio-One, Frickenhausen, Germany) at 37°C, 5% CO_2. Depending on the experiment, wells were pulsed at day 0, 2 and 6 cells for 15 hours with 1 µCi of methyl-^{3}H-thymidine (Hartmann Analytic, Braunschweig, Germany), and incorporation was measured by β-scintillation counting (Wallac 1450, PerkinElmer, Rodgau-Jürgesheim, Germany) (Suppl. Fig. 1A). 1 µg/ml of PHA-L (Sigma, St. Louis, USA) was used as positive control.

HLA-DR15 haplotype-derived self-peptides were either tested in pools or individually at a 2 µM concentration (10 µM total pool concentration) in 7-day proliferation assays. Stimulatory indices (SI) were calculated as SI = mean cpm (counts per minute) (peptide) / mean cpm (unstimulated). Statistical analysis was performed using a student's t test (two-tailed, unpaired) using Prism 5.0 software (GraphPad, La Jolla, USA). Analysis of the mean standard devations has been verified with tests for the homogeneity of variance (Fligner-Killeen, Bartlett, Levene), which have been performed using R (v 2.15.1) with libraries stats and car.

HLA restriction

CD4+ T cells were negatively enriched from thawed PBMCs of HLA-DR15+ HD donors using the IMag system (BD Biosciences, Heidelberg, Germany). 25.000 CD4+ T cells (purity > 95%) were co-cultured for 3 days with 6.5 x 10^4 irradiated (200 Gy) BLS cells in AIM-V serum-free medium. Restriction was tested with BLS cells transfected with a single HLA-class II molecule, DRB1*15:01 (DR2b - DRB1*15:01, DRA*01:01), DRB5*01:01 (DR2a - DRB5*01:01, DRA*01:01), DQw6 (DQA1*01:02, DQB1*06:02). BLS cells were kindly provided by G. Nepom and W. Kwok (University of Washington, Seattle, USA). Proliferation was assessed as described above.

Blocking of autologous proliferation

Autologous T cell proliferation was blocked by incubating PBMCs with anti-HLA-class I (HLA-A, -B, -C; w6/32) and anti-HLA-DR (L243) antibodies at the depicted concentrations and compared to a purified mouse IgG2a$_\kappa$ low endotoxin, azide-free isotype control (BioLegend, MOPC-173, San Diego, USA) in a 3- and 7-day proliferation assay.

Flow cytometry analysis

Samples were thawed, Fc-blocked with mouse IgG and directly stained for surface expression using following antibodies: anti-CD3 (PE-Cy7, UCHT1, eBioscience, San Diego, USA), -CD4 (APC, RPA-T4, eBioscience), -CD8 (PacificBlue, DK25, Dako, Glostrup, Denmark), -CD14 (PacificBlue, MφP9, BD Pharmingen, San Diego, USA), -CD19 (PacificBlue, HIB19, BD Pharmingen), -CD303 (APC, AC144, Miltenyi Biotech, Bergisch Gladbach, Germany), -panHLA-class II (DR, DQ, DP) (FITC, Tü39, BD Pharmingen). Analyses were performed on a LSRII (BD Biosciences) flow cytometer using FACS Diva 5.0 software.

Elution of HLA-presented peptides

HLA-bound peptides were obtained by immunoprecipitation of HLA molecules from the BLS cell expressing only DR2a or DR2b according to standard protocols (Falk *et al.*, 1991) using the DRB5*01:01-specific antibody (clone: H0596) for DR2a and the DRB1*15:01-specific antibody (clone: H0427A) for the DR2b cells. Both were provided by Jar-How Lee (One Lambda Incorporation, Canoga Park, USA). This was followed by a second HLA-precipitation by the pan-HLA-DR specific antibody L243 for each cell lysate.

About 3 ml cell pellet per cell line were gently shaken in 3 ml PBS containing 1.2% (w/v) CHAPS in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Applied Science) for 1 hour followed by sonication of the lysate on ice with subsequent shaking for 1 hour. The lysate was centrifuged and the supernatant was passed through a 0.20 µm sterile filter. Subsequently, HLA molecules and bound peptides were isolated with the solid-phase bound monoclonal antibody by immunoaffinity chromatography (overnight at 4°C). HLA ligands were eluted with 0.2% trifluoroacetic acid, ultrafiltrated through a 10-kDa cut-off Amicon

ultracentrifuge filter, and lyophilized.

Liquid chromatography-mass spectrometry

Lyophilized samples were resuspended in 30 µl of solvent A (0.1 (v/v) formic acid in 2% (v/v) acetonitrile) and loaded onto a C_{18} precolumn (Dionex) for concentration and desalting with a flow rate of 20 µl/min. The precolumn was placed in line for separation by a 250 x 0.075 mm fused silica microcapillary column packed with C_{18} reversed-phase material (Acclaim PepMap 100, Dionex). A binary gradient of 0 to 55% solvent B was performed within 120 minutes, applying a flow rate of 300 nl/min.

The eluted peptides were analyzed by electrospray ionization (ESI) mass spectrometry on a linear trap quadrupole (LTQ) Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to nanoLC-2D (Eksigent) nano-HPLC system via a nanospray-ESI source. A gold-coated fused-silica glass capillary Picotip™ Emitter (SilicaTip™, FS360-20-10 Coating P200P, New Objective) was used for the introduction into the nanospray-ESI source. The heated capillary temperature and spray voltage were held at 200°C and 1.9 kV, respectively, creating an electrospray between the Picotip and the transfer capillary resulting in a sample flow rate of 300 nl/min. Parent ions were measured in the Fourier transform (FT) Orbitrap analyzer. Fragment ions were generated by collision-induced dissociation (CID) with normalized collision energy of 35 V and recorded in the LTQ. The activation time for CID was 30 ms. Mass spectrometry-survey scans were acquired at a resolution of 60 000 at 400 m/z. The “lock mass” option (Olsen *et al.*, 2005) was enabled for the FT-mass spectrometry scans using the 445.120025 m/z ion for real time internal calibrations. The 5 most abundant parent ions from the FT survey scan were selected for fragmentation and excluded from further sequencing for 90 s. The mass

spectrometer was operated in positive ion mode and parent ions with an unknown or higher charge state than 3 were excluded from tandem mass spectrometry.

Mass spectrometrical data analysis

Tandem mass spectrometry data were analyzed with the Thermo Proteome Discoverer 1.1 (Thermo Fisher Scientific) software employing Mascot v2.2 (Matrix Science) as a search engine for peptide identification using a target-decoy database search in the Swiss-Prot protein sequence database (*homo sapiens,* release, 2010_10). For database searches a mass tolerance of 5 ppm were used for FT-mass spectrometry scans whereas 0.5 Da were accepted for LTQ-MS/MS scans. The false discovery rate was set to ≤ 1%. Phosphorylation of serine (S), threonine (T) and tyrosine (Y) as well as oxidation of methionine to methionine sulfoxide (m) were enabled as dynamic modifications. The software was allowed to group spectra if the retention time (RT) difference did not exceed 24 s.

Peptide library generation and *in silico* peptide binding prediction

122 overlapping (5 amino acid overlap) 15- and a few 16-mer peptides from DRB1*15:01, DRB5*01:01, DRA*01:01, DQA1*01:02, DQB1*06:02 (http://hla.alleles.org) were synthesized and provided by pe (peptides & elephants GmbH, Potsdam, Germany). DRβ-chain-derived peptides with identical sequences were only synthesized once. Lyophilized peptides were resuspended in 100% DMSO and stored at -80°C.

In silico peptide binding predictions were performed by the consensus approach provided by the Immune Epitope Database (www.iedb.org, (Wang *et al.*, 2008)). Gene Ontology annotation analysis of biological pathways was performed

with the DAVID functional annotation analysis webpage (http://david.abcc.ncifcrf.gov/).

RESULTS

Autologous proliferation is altered in multiple sclerosis patients

We first characterized and compared in a standard thymidine incorporation assay the proliferation of PBMCs from 69 untreated multiple sclerosis patients and 36 healthy donors (HD; for demographics see Suppl. Table 1) over seven days in serum-free media. Below, we will refer to this condition in the absence of a stimulus as autologous proliferation. Since there is no background control and a stimulation index cannot be calculated, we expressed the **strength of proliferation** by the mean counts per minute (cpm) of all wells (Fig. 1A) and the **variation or heterogeneity** by the mean, amongst multiple donors within each group, e.g. multiple sclerosis or controls, of the standard deviation of all wells within each donor (Fig. 1B; schematic depiction in Fig. 1C). PBMCs from multiple sclerosis patients incorporated significantly more thymidine (mean cpm ± SEM, 5337 ± 548) than HD (3606 ± 503, P = 0.04), while no differences in strength of proliferation were observed after PHA stimulation (Fig. 1A). PBMCs from multiple sclerosis patients not only incorporated more thymidine, but also the variation between wells was significantly higher or heterogeneous (SD ± SEM, 2099 ± 217) than in HD (1422 ± 180, P = 0.04) (Fig. 1B), and again no differences in heterogeneity were observed after PHA stimulation (data not shown). Some wells from multiple sclerosis patients proliferated as vigorously as usually observed upon stimulation with nominal antigen (Fig. 1C), suggesting that some T cells from multiple sclerosis patients are activated in the absence of a stimulus. We then compared patients with different forms of multiple sclerosis, i.e. clinically isolated syndrome (CIS), a pre-stage of multiple sclerosis, relapsing-

remitting multiple sclerosis (RR-MS) in remission (RR-MS-remission) or in exacerbation (RR-MS-relapse), secondary-progressive multiple sclerosis (SP-MS) and primary-progressive multiple sclerosis (PP-MS). The differences of the strength of proliferation were similar in the subgroups, while the heterogeneity measure was higher for CIS- and RR-MS patients in relapse (Suppl. Fig. 1B). In order to exclude that a leuko- or lymphopenic condition of the donors/patients with increased autologous proliferation elicited homeostatic proliferation, i.e. growth of T lymphocytes to fill an empty or partially emptied niche, we performed differential blood counts on all donors. Only two HD showed lymphocyte counts slightly below normal values, and there was no correlation between peripheral blood white cell- or lymphocyte counts and the degree of autologous proliferation (data not shown).

Increased autologous proliferation is related to the multiple sclerosis-associated DR15 haplotype

We next addressed the involvement of the HLA-DR15 haplotype in the above findings. As shown in Figure 2A, the strength of autologous proliferation (mean cpm) was higher in DR15+ individuals than in DR15- when comparing all studied individuals (left panel) and also in multiple sclerosis patients versus HD, although differences were only significant for DR15- HD versus DR15+ multiple sclerosis patients (second panel from left; $P = 0.045$). The differences were more pronounced for the proliferation heterogeneity (mean SD) of all studied DR15+ versus DR15- individuals (second panel from the right; $P = 0.004$) and for both DR15- versus DR15+ HD (right panel; $P = 0.047$) and DR15- versus DR15+ multiple sclerosis patients (right panel; $P = 0.036$) indicating that autologous proliferation strength and -heterogeneity increase with disease- and HLA-DR15 status, which is particularly clear from the “single-well” view of PBMCs (Fig. 2B). Here, each column represents a

single donor with all seeded wells. Each well is represented by a white dot. Donors were sorted by the well with the highest cpm count and according to disease and HLA status. Supplementary Table 1 summarizes the results of Figure 2A and 2B.

Characterization of autologous proliferating cultures

In order to characterize better, which cell population in the peripheral blood mononuclear cells is responsible for autologous proliferation we determined the percentage of CD4+, CD8+ and memory CD4+ T cells before and after 7 days of in culture. As expected autologous proliferation was primarily mediated by CD4+ T cells, since their percentages were high at the time of starting the assay and increased further until day 7 (Fig. 3A, left panel), while the initially low numbers of CD8+ T cells dropped (Fig. 3A, third panel from left) indicating that under uninhibited culture conditions CD8+ T cells did not proliferate to a significant extent. Further, we did not observe major changes regarding the naïve/memory CD4+ T cell population composition (Fig. 3A, second panel). However, the slight but consistent decrease of CD4+CD45RO+ T cells indicates that autologous proliferation is mediated to a larger extent by naïve-, i.e. CD4+CD45RO-, then by memory T cells.

Next we wanted to address the potential contribution of the level of baseline HLA-DR expression or its upregulation during the culture period. As shown in Fig. 3A (right panel), DR expression on B cells increased significantly, supporting a putative role of HLA-DR in the above phenomena (Fig. 3A, right panel). We then asked whether a higher baseline HLA-DR/DR15 expression on APCs is a consequence of for example a proinflammatory environment in multiple sclerosis and then causes increased homeostatic proliferation. Toward this aim we examined the HLA-class II expression (DR, DP and DQ) on plasmacytoid dendritic cells, monocytes and B cells in HD and multiple sclerosis patients homozygote or heterozygote for DR15 as

indicated by Cella et al. (Cella *et al.*, 1997). HLA-class II expression did not differ significantly between donors with the exception of lower levels of HLA-DR expression on monocytes of multiple sclerosis patients (Fig. 3B, middle panel). Also, there was no DR-related gene dosage effect in DR15 homozygous versus heterozygous multiple sclerosis patients or HD. Finally, blocking antibodies against HLA-class II alone (Fig. 3C, left panel) and against both HLA-class I and -class II (Fig. 3C, right panel) reduced the autologous proliferation in a dose-dependent manner both at day 3 and day 7 (Fig. 3C). Together these data indicate that interactions between CD4+ T cells and their TCR with self HLA-class II molecules are involved in autologous proliferation, that B cells may play a role in this phenomenon *in vitro* and that different levels of HLA-class II expression at baseline are unlikely to be involved.

Elution of HLA-DR-derived self-peptides from the DR alleles expressed in the multiple sclerosis-associated DR15 haplotype

The above observations point at an involvement of the two DR15 alleles, DRB1*15:01 (DR2b) or DRB5*01:01 (DR2a) and the self-peptides they display in increased autologous T cell proliferation in DR15+ multiple sclerosis patients. Multiple factors could contribute to this observation including a genetically determined lower activation threshold of T cells in multiple sclerosis and a TCR repertoire that is shaped in DR15+ individuals by central tolerance mechanisms, which has higher affinity for DR15/self-peptide complexes. With respect to the latter, structural features of the DR molecules, i.e. their contact surfaces with the TCR of CD4+ T cells, or the spectrum of bound self-peptides or both could play a role. To address the latter aspect we next examined the nature of self-peptides and their potential role in increased autologous proliferation by characterizing self-peptides that are bound to DR2a and DR2b by peptide elution and sequencing by mass

spectrometry (schematically shown in Fig. 4A). In a prior study, we had employed Epstein-Barr virus-transformed B cell lines that co-expressed all alleles of the DR15 haplotype using a monoclonal antibody against monomorphic determinants of DR alleles (Vogt *et al.*, 1994). To focus on DR2a and DR2b directly, we here used bare lymphocyte syndrome (BLS) patient-derived B cell lines that had been transfected with either DR2a or DR2b and did not express any other HLA-class II molecule. Furthermore, for precipitation of DR2a and DR2b molecules we used allele-specific monoclonal antibodies against DR2a or DR2b, respectively. Due to the lower precipitation efficiency of the latter IgM antibodies, a second sequential immunoprecipitation was performed with the L243 antibody. Since BLS transfectants only express one class II allele, the peptide elution presumably underestimates the relative presence of HLA-class II-derived peptides by limiting the number of available class II molecules. We eluted and identified a total of 154 peptides from DR2a and DR2b molecules (Fig. 4A; Suppl. Table 2), and 7.14% (w/o considering multiple protein hits) of these peptides were derived from DRα (DRA1*01:01) or the β chains of DR2b (DRB1*15:01) or DR2a (DRB5*01:01). Hence, HLA-class II molecule-derived self-peptides form a substantial fraction of the spectrum of self-peptides that are bound to the multiple sclerosis-associated DR15 alleles. Biological pathway gene ontology annotation of the eluted peptides demonstrated that many of them are assigned to the antigen presentation pathway (Suppl. Table 3). Herewith we confirm and broaden our earlier results that HLA-class II-derived self-peptides make up a considerable portion of the peptide spectrum presented by DR2a and DR2b (Vogt *et al.*, 1994).

Reactivity to HLA-DR15 haplotype-derived self-peptides is related to expression of the haplotype itself

The above data indicate that HLA-derived self-peptides probably participate in autologous proliferation and also in T cell activation/stimulation beyond autologous turnover. To address this point we generated a HLA-DR15 haplotype-derived self-peptide library consisting of 15-mer peptides with 5 amino acids overlap spanning the whole HLA-DR15 haplotype protein sequence (Fig. 4B). The resulting 122 peptides were derived from 5 proteins, i.e. the polymorphic chains HLA-DRB1*15:01 (DR2b), -DRB5*01:01 (DR2a), the conserved HLA-DRA*01:01, and the HLA-DQw6 molecule consisting of HLA-DQA1*01:02 and -DQB1*06:02 (Fig. 4B, Suppl. Table 4). HLA-DRβ chains share most of their sequences and are only polymorphic in certain areas, and therefore peptides derived from shared sequences were only synthesized once (Fig. 4B and C).

Since the relative genetic risk conferred by the multiple sclerosis-associated HLA-DR15 haplotype correlates not only with DR15 carrier status, but also with its dose, i.e. homo- versus heterozygosity (Barcellos *et al.*, 2003; Cournu-Rebeix *et al.*, 2008) we used the entire HLA-derived peptide library, i.e. 24 pools of 5 peptides each, to stimulate PBMCs from multiple sclerosis patients/HD (20 donors each) that were stratified according to DR15 homo- and heterozygosity (Fig. 5A). Since large genetic studies had indicated that there are also HLA-DR alleles that are “protective” for multiple sclerosis, e.g. the HLA-DR14 haplotype (Ramagopalan *et al.*, 2007), we included a HLA-DR15/”protective” cohort (HLA-DRB1*14, -DRB1*11), which should have the lowest risk for multiple sclerosis development regarding HLA background. We did not find these “protective” DR alleles among our multiple sclerosis patients, and therefore only tested HD. Figure 5A depicts the % of wells with an SI > 2.0 of all peptide pools tested for the different donors. While the differences did not reach statistical significance (Fig. 5A, left panel), there is a clear trend that the reactivity increases from protective DR allele carriers to DR15 heterozygous- to homozygous

DR15 carriers. Due to the higher background/autologous proliferation in multiple sclerosis patients (see above), which affects the calculation of the stimulatory index (SI), effects are more pronounced in HD. In addition, we tried to capture not only proliferating cells according to standard criteria, i.e. surpassing background proliferation by at least a factor of two (SI > 2), but also those showing a “low-grade” proliferation (defined as SI > 1.4). Since we added here nominal peptide in distinction to the above unstimulated condition, which we referred to as autologous proliferation, we will from now on refer to the peptide-induced “low-grade” proliferation as “tonic” stimulation. As shown in Fig. 5A (middle panel), the tonic stimulation also increases in relation to DR15 haplotype and homozygosity.

Potential role of HLA-DR15 haplotype-derived self-peptides in autologous T cell proliferation

Next, we assessed the stimulatory- or inhibitory potential of the HLA-derived self-peptides (Fig. 5B). Selected individual HLA-DR15-derived self-peptides were tested after analyzing the proliferation of 35 Donors (20 multiple sclerosis, 15 HD) to the 24 pools of self-peptides (Suppl. Fig. 2). Additionally, since they are most likely to be presented *in vivo*, we also tested two peptides, which had been eluted. Peptide 8 (SDVGEFRAVTELGRP) and its DR2a-homologue had been eluted twice, by Vogt et al. (Vogt *et al.*, 1994) and again from DR2a in the present study. Peptide 54 (EEFGRFASFEAQGAL) was eluted from HLA-DR2b. Like peptides 14, 15, 20, 45, both elicited strong T cell proliferation (Fig. 5B, left panel). These experiments demonstrate that distinct DR15 haplotype-derived self-peptides are stimulatory to bulk PBMCs, while other peptides seem to inhibit activation or proliferation (SI > 2.0). As described in Figure 5A, we used the thresholds of SI > 2.0 and > 1.4 respectively to discriminate between activation and tonic proliferation. When looking at one

specific peptide as example, peptide 45, 12.5% of all wells proliferated with SIs > 2.0 (Fig. 5C) and 66% with SIs > 1.4 (not shown) indicating that a large number of wells responds with a tonic proliferation pattern, and a fraction of cells is fully activated. Interestingly, peptide 45 showed a marked T cell response despite originating from the inhibitory pool number 9 supporting the consideration that inhibitory and stimulatory peptides within each pool interact, consequently explaining the low overall proliferative response to the peptides pools (Suppl. Fig. 2).

We further asked if the stimulatory HLA-DR15-derived self-peptides could, if pooled together, evoke a stable T cell proliferation and if so, which concentrations are sufficient to induce proliferation. For this purpose different doses between 0.1 and 20 μM of a pool of five strongly stimulatory peptides were used. Peptide 54 had been eluted, peptides 8 and 20 were analogues of eluted peptides, and peptides 14 and 45 demonstrated the strongest tonic proliferation stimulus in the above proliferative assays with individual peptides. As shown in Fig. 5C, the pool of stimulatory peptides led to full stimulation and also tonic proliferation over a wide range of concentrations with a peak at 1.0 μM (Fig. 5C).

Next, we used the above five stimulatory peptides and examined the HLA-class II restriction using purified CD4+ T cells and BLS transfectants expressing either DR2b, DR2a or DQw6. Most of the peptides elicited their stimulatory effect when presented by HLA-DR2a (Fig. 5D, dark grey). Interestingly, when they were added to APCs expressing HLA-DQw6, most peptides showed inhibitory rather than stimulatory effects.

Characterization of polymorphic HLA-DRβ-derived peptides

Finally, we compared corresponding peptides derived from the polymorphic regions of the two DRβ chains (Fig. 6A; see also Fig. 4C). We found significant

differences in 5 of 10 pairs (Fig. 6A). In 4 of the 5 pairs DR2b-derived peptides (Fig. 6A, light grey) were more stimulatory, while we found the reverse, i.e. that the DR2a-derived peptide was more stimulatory, only for peptide 44 versus peptide 22. Peptide 4 (DTRPRFLWQPKRECH, DR2b-derived) gave the strongest proliferative response of all polymorphic peptides. Figure 6B summarizes all allele-specific DRβ chain-derived peptides, which we compared in Figure 6A. In order to understand better, if these peptides bind to either of the two DR15 molecules, DR2a and DR2b, or to DQw6, we performed well-established *in silico* peptide binding predictions. The predicted binding affinities (% consensus rank; low values indicate better binding; highest affinity highlighted in green) are summarized in Figure 6B, and we further mention if the respective peptide was eluted from DR15 molecules in this or our prior study (Vogt *et al.*, 1994). Table 2 additionally analyzes peptides that have been eluted or shown to be stimulatory in our experiments. Both tables (Figure 6B and C) depict the peptide origin and the probability of being presented by an HLA-class II molecule of the DR15 haplotype. It is interesting to note that, while more of the stimulatory polymorphic peptides derive from the DR2b allele, the predicted binding affinities of these peptides are higher for binding to DR2a and, consistent with this, the peptides have been eluted from DR2a.

DISCUSSION

Despite substantial progress in immunology we still know remarkably little about how certain HLA-class II alleles contribute to autoimmune diseases (Gronski *et al.*, 2004; McFarland and Martin, 2007). HLA-DR15 as major risk factor for multiple sclerosis is one of the most striking examples for this notion. The mechanisms as to how HLA-DR15 shapes and maintains an autoreactive T cell repertoire that leads to central nervous system (CNS) autoimmunity are still elusive despite longstanding

efforts by a number of groups including our own, which focused primarily on studying the antigen specificity, function and HLA restriction of autoreactive T cell clones. In the present study we explored a hypothesis that had been discussed earlier in the context of autoimmune liver disease (Burroughs *et al.*, 1992) and oligoarticular juvenile idiopathic arthritis (Massa *et al.*, 2002) but not for multiple sclerosis so far. These studies postulate that self-peptides derived from and presented by abnormally expressed HLA-class II molecules potentially mimic microbial/viral peptides and may serve as target for the autoimmune T cell responses. Based on the consideration that interactions between HLA-class II molecules and the TCRs of CD4+ T cells are besides IL-7 the most important factor to keep peripheral T cells alive, we examined the functional involvement of the HLA-DR15 haplotype in shaping and/or maintaining the peripheral T cell repertoire in multiple sclerosis via autologous proliferation. It has to be clarified, however, that we cannot assess by our simplified in vitro system the complex in vivo interactions that lead to T cell repertoire maintenance. Different from the steady-state in vivo homeostatic proliferation, i.e. in the absence of lymphopenia, which involves HLA/MHC/self-peptide complexes and/or IL-7 depending on the cell type (Baccala and Theofilopoulos, 2005; Cox *et al.*, 2005), our assay system most likely depicts a combination of autologous activation and subsequent proliferation in the absence of any stimulus. As stated above, this setting is different from the well-examined autologous mixed lymphocyte reaction, where antigens that are generated by apoptosis of the irradiated stimulator cells are recognized by autologous T cells (Amel Kashipaz et al., 2002), which probably also explains the divergent results between autologous mixed lymphocyte reaction in multiple sclerosis, i.e. decreased proliferation (Hafler et al., 1985), and our observation of increased autologous proliferation.

With advanced age thymic output decreases, and hence compensatory peripheral homeostatic proliferation becomes more important for maintaining a functional T cell repertoire (Naylor *et al.*, 2005). Previous studies have described earlier thymic involution as well as reduced generation of CD4+ recent thymic emigrants in multiple sclerosis (Duszczyszyn *et al.*, 2010). Here, we show elevated autologous T cell proliferation in patients with multiple sclerosis compared to HD in our experimental *in vitro* system. The autologous proliferation is more heterogeneous in that a substantial fraction of cells shows signs of full activation in multiple sclerosis patients, and it is related to the multiple sclerosis-associated HLA-DR15 haplotype. Based on the importance of HLA-class II molecules, presented self-peptides, and the TCR avidity (Sprent and Surh, 2011) for this process, we assume that the HLA-DR15 haplotype is involved in altered homeostatic T cell proliferation in multiple sclerosis. Since the multiple sclerosis patients of this study were not lymphopenic, i.e. did not have a relative depletion of the lymphocyte niche, it appears that lymphocytes are not numerically reduced, but rather that their repertoire is more narrow with respect to different specifities, but enriched for T cells with higher affinity for self-peptide. Such a repertoire is then expected to be activated easier in an autologous setting, which is simulated by our *in vitro* system.

Although immune mechanisms exist that sustain a T cell repertoire of great diversity (Min *et al.*, 2004) previous studies have reported that the peripheral maintenance of naïve T cells is not random (Rudd *et al.*, 2011). Under homeostatic conditions TCR affinity/avidity appears to regulate T cell survival (Kieper *et al.*, 2004). In line with these concepts, we have previously shown that the presentation of self-peptides by mature DCs can lead to a state of preactivation of T cells (Kondo *et al.*, 2001). Together, these studies emphasize that homeostatic/autologous proliferation supports mechanisms of selection and preferential activation of certain parts of the T

cell repertoire. Therefore, the heterogeneous autologous proliferation with some cells showing a tonic response and others signs of full activation could indicate a process of selection through predominant proliferation of certain sets of T cells expressing TCRs with higher avidities for self-MHC/self-peptide complexes.

During T cell activation the TCR contacts both antigenic peptide and HLA-backbone. The two alpha-helices of the HLA-backbone represent over 60% of the TCR:MHC/peptide interaction surface (Sundberg *et al.*, 2007). When dissecting the trimolecular complex (TCR:MHC/peptide), the following considerations are important. First, the higher the contribution of the MHC backbone to overall avidity the higher the number of peptides that can be recognized by the TCR. Second, since T cell activation (or prevention of death) is related to TCR affinity, selection is a consequence of homeostatic T cell proliferation. Third, the composition of self-peptides that are presented by a given HLA molecule is constrained by structural aspects of its binding pockets. Fourth, the presenting HLA molecule itself, which passes through the main processing compartments, is an important source of peptides that are displayed in its own binding pockets (Dengjel *et al.*, 2005). Therefore, HLA haplotypes present a haplotype-specific diverse set of self-peptides, which probably participates in shaping the peripheral T cell repertoire. Since our *in vitro* studies show that the heterogeneity of proliferation was enhanced by the HLA-DR15 haplotype, we hypothesize that either the structural configuration of the HLA backbone, the self-peptides presented by the HLA-DR15 haplotype, or a combination of both are at least in part responsible for the observed proliferation patterns.

Most of the time surface HLA-class II molecules are loaded with self-peptides (Schild *et al.*, 1990). To analyze the potential role of self-peptides derived from the multiple sclerosis-associated HLA-DR haplotype and thus address some of the above possibilities, we eluted peptides presented by the HLA-DR15 haplotype.

Consistent with prior work including ours' (Vogt *et al.*, 1994; Dengjel *et al.*, 2005) we identified self-peptides from various sources and mostly from processing- and antigen presentation-related molecules. Among the latter a substantial fraction was derived from molecules of the HLA-DR15 haplotype itself. Our data indicate that HLA-derived self-peptides play an important role in the observed HLA haplotype-specific differences in autologous proliferation and that they can promote T cell proliferation. By using systematically generated sets of peptides spanning all DR- and DQ α- and β chains, we demonstrate that relatively high frequencies of peripheral T cells recognize certain HLA-derived self-peptides. While T cell proliferation is often tonic, full activation is not a rare event even without any antigenic stimulus in PBMCs from multiple sclerosis patients. These data indicate that HLA-DR15-derived self-peptides are able to provide a low-level stimulus or fully activate parts of the peripheral T cell repertoire. Furthermore, the observed HLA-derived peptide reactivity correlated with the genetic risk conferred by the HLA-DR haplotype. In individuals, who are haploidentical with respect to HLA-class II type, the combined effects of stimulatory and inhibitory self-peptides derived from multiple sclerosis risk-conferring and/or -lowering HLA alleles probably participates in setting the activation thresholds of T cells.

Positively selecting self-peptides in the context of CD4+ T cells have recently been described in murine models (Lo *et al.*, 2009), and the most important conclusions from these studies were the high specificity in the recognition of positively selecting ligands and that the same self-peptides can support peripheral homeostatic proliferation or serve as coagonists in the recognition of nominal ligand (Krogsgaard *et al.*, 2007; Wucherpfennig and Gagnon, 2009). Since the number of self-peptides that are involved in thymic selection processes is considered to be small, and many of these are presented both on thymic- and peripheral APCs, they

are probably relevant in both contexts, i.e. during central selection and peripheral homeostasis. Although thymic T cell repertoire selection appears to depend primarily on TCR:MHC/peptide affinity, the peripheral maintenance and selection is determined by TCR cross-reactivity (Hao *et al.*, 2006). Regarding the latter, the TCR cannot distinguish between self- and non-self peptides (Ashton-Rickardt *et al.*, 1994; Krogsgaard *et al.*, 2007), and therefore self-peptides presumably are a major factor in shaping and maintaining the composition of the peripheral T cell repertoire. Since HLA molecules bind different sets of peptides based on the structural requirements of their binding pockets (Falk *et al.*, 1994), different self-peptide repertoires will be presented according to haplotype. These factors together with the structural characteristics of the exposed surface of HLA molecules most likely lead to HLA-haplotype-specific shaping of the T cell receptor repertoire.

Besides these structure-related possibilities our data point at yet another one, which involves peptides derived from the non-polymorphic or polymorphic stretches of HLA molecules as antigens themselves. Interestingly, an earlier study on the role of polymorphic regions of different DRB1* alleles including DRB1*15:01 observed significant multiple sclerosis-association of the amino acids proline at position 11, arginine at position 13, and alanine at position 71 of HLA-DRB1*15:01 (Zipp *et al.*, 2000). This study did not examine the DRB5* allele nor the abovementioned structural contributions or role of a polymorphic DR-derived peptide as antigen. In our present study, HLA-derived peptide number 4 contains both proline at position 11 and arginine at position 13, and therefore this peptide could contribute to increased autologous proliferation by shaping the binding pocket of DR2b, or conversely it could be an antigen itself. Peptide 4 was the most stimulatory of the polymorphic areas (see Fig. 6B), while the homologous peptide 30 derived from the same area of DRB5*01:01 was not stimulatory.

If one attempts to link these findings to multiple sclerosis, it is currently believed that autoreactive T cell responses in multiple sclerosis are directed against myelin proteins including myelin basic protein (MBP) (Sospedra and Martin, 2005). Early studies by others and us have found MBP-specific T cells in multiple sclerosis patients, but also in healthy controls (Martin *et al.*, 1990; Ota *et al.*, 1990; Pette *et al.*, 1990). Despite higher precursor frequencies in multiple sclerosis patients in some studies this observation at first seemed to argue against a role of these cells in multiple sclerosis, but later studies not only described higher frequencies, but also other potentially relevant characteristics such as higher functional TCR avidity against several myelin peptides derived from MBP, myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) (Bielekova *et al.*, 2004), populations that are independent of costimulation (Markovic-Plese *et al.*, 2001), signs of activation (Bielekova *et al.*, 1999) and enrichment in the naïve CD4+ T cell compartment (Muraro *et al.*, 2000). An interesting question is if the increased autologous turnover that we describe here, also leads to conversion to memory phenotype in autoreactive T cells, which would explain the increased numbers of MBP-specific memory cells in multiple sclerosis patients (Burns *et al.*, 1999). In line with this speculation, investigations of the CD8+ T cell compartment show that T cell numbers and diversity decrease with age, surviving T cells undergo faster rates of homeostatic proliferation, are selected for high TCR avidities, and preferably acquire “memory-like” phenotype (Rudd *et al.*, 2011). The relatively early thymic involution that was described in multiple sclerosis (Duszczyszyn *et al.*, 2010) could lead to an increased demand for compensatory peripheral homeostasis in the CD4+ compartment as observed in the autologous proliferation setting. A combination of the presented HLA-haplotype-specific self-peptides and structural features of the HLA-DR15 haplotype may lead to alterations in the overall strength and quality of

proliferation with the consequence of selecting an “autoimmune-prone” T cell repertoire.

Another important aspect is the question if only DRB1*15:01 or also DRB5*01:01 determines multiple sclerosis risk. A number of studies indicate the former, i.e. that risk is only conferred by DRB1*15:01 (Caillier *et al.*, 2008). We favor that both alleles contribute jointly to multiple sclerosis risk based on the similarities of the peptide binding motifs (Vogt *et al.*, 1994; Wucherpfennig *et al.*, 1994), which lead to presentation of similar peptides including the immunodominant MBP peptide$_{83\text{-}99}$, which can be presented equally well by both alleles (Martin *et al.*, 1991; Valli *et al.*, 1993; Wucherpfennig *et al.*, 1994). Also, humanized transgenic mice expressing a multiple sclerosis patient-derived MBP$_{83\text{-}99}$-specific TCR and DR2a (DRB5*01:01) develop spontaneous experimental autoimmune encephalomyelitis (Quandt *et al.*, 2012) as do transgenic mice expressing a MBP$_{84\text{-}102}$-specific TCR and DR2b (DRB1*15:01) (Madsen *et al.*, 1999). Furthermore, myelin-specific- and virus-specific T cell clones, which recognize the same and different peptides in the context of both DR2a and DR2b, have been described (Lang *et al.*, 2002; Sospedra *et al.*, 2005), and that their cross-restriction leads to higher antigen avidity (Sospedra *et al.*, 2006; Yousef *et al.*, 2012). Here, we observe that the majority of HLA-derived polymorphic peptides that stimulate T cell proliferation are derived from DR2b (e.g. peptides 4 and 8), however, when examining their peptide binding to the two DR molecules, both show stronger affinity, are restricted by and have been eluted from DR2a (Fig. 6). We observed the reverse with peptide 45. Finally, we found non-polymorphic peptides, i.e. peptide 54 derived from DRA1*, which is predicted to bind better to DR2b and which we had also previously eluted from DR2b (Fig. 6C), although the bulk CD4+ T cells recognize the peptide when presented by DR2a (Fig. 6A). These data do not answer definitively the above question, which of the two alleles is more relevant for

shaping an autoimmune-prone T cell repertoire in DR15+ multiple sclerosis patients, but they support the notion that both are involved.

Taken together, our observations are in line with previous reports describing early thymic involution and repertoire narrowing in patients with multiple sclerosis. The subsequent demand for an increased peripheral maintenance and the accompanying effects on TCR repertoire selection are reflected not only in the enhanced-, but also in a more heterogeneous proliferation pattern. Further, we demonstrate that the HLA-DR15 haplotype-derived self-peptides and/or structural features of the two HLA-DR molecules of the DR15 haplotype are likely involved in increased autologous proliferation. It remains to be shown if T cells that are expanded by autologous proliferation have the propensity to cross-react with myelin- or CNS autoantigens as indicated by (Cai and Hafler, 2007), can be activated by peptides from foreign agents and if the autologous proliferation leads to preferential differentiation into proinflammatory T cells. There are probably additional factors that contribute to the proliferation patterns including cytokine receptors, cytokines, costimulatory molecules and their ligands, CD4-HLA-class II co-receptor interactions, and integrin/adhesion molecules, which may all be involved in regulating the threshold for T cell activation, -expansion or functional differentiation. Interestingly, single nucleotide polymorphisms of a number of these (IL7RA, IL2RA, IL7, CD58, CBLB, CD40, IL12B, IL22RA2, and others) have been found to be involved in multiple sclerosis risk (Sawcer *et al.*, 2011). Based on our observations and the genetic data, we propose the following model. The two DR alleles in the DR15 haplotype and as yet unknown self-peptides select by central tolerance mechanisms a TCR repertoire that has the inherent propensity to recognize CNS autoantigens and lead to a CNS-specific autoimmune T cell response. Positively selected T cells are maintained and/or expanded by peripheral homeostatic proliferation, which again

involves the two DR15 alleles and according to our data among the spectrum of self-peptides also those derived from the DR15 haplotype themselves. Both a genetically predetermined lower activation threshold and certain environmental triggers then finally lead to full activation and expansion of CNS-autoreactive T cells, their proinflammatory differentiation and eventually the development of multiple sclerosis.

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FIGURE LEGENDS

Figure 1. Comparison of autologous proliferation of peripheral mononuclear cells. Significant differences in proliferation strength (A) (mean cpm ± SEM) and heterogeneity (B) (standard deviation ± SEM) are observed between multiple sclerosis (MS) and healthy donors (HD) at day seven (* indicating *P*-value of < 0.05). (A) Right bar graph depicting the PHA positive control. (C) Example of one multiple sclerosis donor showing all unstimulated wells over a seven-day proliferation assay with adjacent scheme explaining measures of proliferation strength and heterogeneity.

Figure 2. Influence of HLA-DR15 haplotype on autologous proliferation. (A) Proliferation strength is shown for multiple sclerosis (MS) and healthy donors (HD) together (left graph) or subdivided into HLA-DR15 haplotype-positive and -negative patients and HD (right graph)(mean cpm ± SEM). (B) Proliferation heterogeneity is

shown for multiple sclerosis and healthy donors together (left graph) or subdivided into HLA-DR15 haplotype-positive and -negative patients and HD (standard deviation ± SEM). (B) Each column represents one donor with dots displaying every single unstimulated well (thymidine incorporation at day 7; * indicating *P*-value of < 0.05, average wells per donor = 22).

Figure 3. T cell subpopulations and HLA-DR expression: (A) FACS analysis of CD4+, CD4+ CD45RO+ and CD8+ surface expression on CD3+ T cells as well as the HLA-DR expression changes on B cells during seven days of unstimulated PBMCs proliferation of 5 HD. (B) HLA-class II expression (median fluorescent intensity; MedFI) on different APCs of HLA-DR15-positive and -negative multiple sclerosis patients (MS) and HD: CD303+ plasmacytoid dendritic cells, CD14+ monocytes and, CD19+ B cells. (C) Blocking of autologous proliferation using anti-HLA class I- and -DR-specific antibodies. Inhibition of proliferation was measured on day 3 and day 7 and is shown as % of uninhibited proliferation.

Figure 4. HLA-DR2a and –DR2b-specific peptide elution and characterization, and HLA-DR15 haplotype-derived self-peptide library. (A) Schematic drawing of peptide elution from BLS cells expressing only DRB1*15:01 (DR2b) or DRB5*01:01 (DR2a), respectively. (B) Pictogram illustrating the composition of the HLA-DR15 haplotype-derived library of self-peptides (15mers). (C) Exon 2 sequences of DRB1*15:01 and DRB5*01:01. Origin of allele-specific DRβ chain-derived peptides and peptides with shared sequences are depicted with single- and red and orange colors.

Figure 5. Proliferation of peripheral blood mononuclear cells upon stimulation with HLA-DR15 haplotype-derived self-peptides. (A) % positive wells tested against 24 pools of HLA-DR15-derived self-peptides. Multiple sclerosis (MS) patients (MS risk n = 10, MS hetero n = 10) and HD (HD risk n = 7, HD hetero n = 8, HD protective n = 5) were stratified according to HLA-DR15 status as depicted by the schematic on the right. Risk indicates homozygosity for HLA-DR*15, hetero indicates HLA-DR*15/*X, and donors with protective alleles carry HLA-DR*15/*14 or -*11 haplotypes. % positive wells with SI > 2.0 (left graph) and SI > 1.4 (right graph). (B) Proliferation of PBMCs to individual HLA-DR15-derived self-peptides (% of wells > SI 2.0, n = 6) grouped by stimulatory, inhibitory and eluted peptides. (C) The most stimulatory peptides from B (peptide 8, 14, 20, 45, 54) were pooled and tested in a dose titration experiment. % wells with SI > 2 and SI > 1.4 are shown (n = 6). (D) Proliferation of isolated bulk CD4+ T cells in response to 5 HLA-derived peptides presented by APCs (BLS transfectants) expressing only DR2a, DR2b, or DQw6 respectively (mean stimulatory index (SI) with SEM using 5 donors, 6 wells each).

Figure 6. Reactivity to HLA-DRβ chain-derived, allele-specific self-peptides. (A) Comparison of 20 peptides derived from the polymorphic HLA-DRβ chains of DRB1*15:01 and DRB5*01:01 (5 donors, 8 wells each; * indicating *P*-value of < 0.05). Scheme depicting origin of allele-specific peptides and shared peptide regions of the DRβ-derived sequence. (C) Comparison of the allele-specific-, DRβ chain-derived peptides. Polymorphic amino acids are marked in red, the highest predicted binding affinity (consensus rank < 20%) to one of the alleles with green shading, and the peptide giving stronger responses in proliferation testing (see Fig. 6B) is indicated by grey shading. (D) Summary of 4 peptides that had been eluted from either DR2a or DR2b.

Suppl. Figure 1. Comparison of peripheral blood mononuclear cell proliferation over a 7-day time course. (A) Proliferation strength (mean cpm ± SEM) is observed between multiple sclerosis patients (MS, n = 40) and healthy donors (HD, n = 14) on day 1, 3 and 7. (B) Proliferation heterogeneity on day 7 is shown for HD (n = 14) and multiple sclerosis patients, which are subdivided into the different multiple sclerosis forms, clinically isolated syndrom (CIS), relapsing-remitting (RR-MS), relapsing-remitting in relapse (RR-MS in R), primary-progress (PP-MS), secondary-progressive (SP-MS) (each n = 8) (standard deviation ± SEM).

Suppl. Figure 2. Proliferation of peripheral blood mononuclear cells upon stimulation with HLA-DR15 haplotype-derived self-peptides. (A) Reactivity to individual pools of the HLA-DR15 haplotype-derived peptide library is shown as mean SI and % of wells SI > 2.0 (20 multiple sclerosis patients and 15 HD). Peptide pools that were selected for further fine specificity analysis are shown in red (stimulatory) or blue (inhibitory).

SUPPLEMENTARY TABLES

Suppl. Table 1: Multiple sclerosis (MS) patient and healthy donor (HD) demographics

	n	mean age ± SD	mean cpm ± SEM	mean SD ± SEM
MS	69	39,83 ± 9,77	5337 ± 548	2099 ± 217
HD	36	42,93 ± 15,72	3606 ± 503	1422 ± 180
male	35	41,7 ± 14,0	4526 ± 751	1824 ± 269
female	70	41,6 ± 11,2	4853 ± 482	1888 ± 197
MS DR15-	22	36,35 ± 10,57	4482 ± 737	1437 ± 178
MS DR15+	47	40,53 ± 9,16	5737 ± 724	2408 ± 298
HD DR15-	15	45,13 ± 16,96	2981 ± 722	1002 ± 203
HD DR15+	21	40,64 ± 15,07	4053 ± 688	1722 ± 257
DR15-	37	41,7 ± 13,8	3874 ± 534	1261 ± 137
DR15+	68	41,5 ± 11,0	5217 ± 549	2196 ± 223

Suppl. Table 2: Peptides eluted from DR2a and DR2b

Eluted from: BLS-DR2a using HLA-DR2a-specific antibody (H0596)

Sequence	Gene ID	Protein	Multiple protein hits	IonScore	Charge	MH+ [Da]	ΔM [ppm]
GYLPNQLFRTF	1653	DDX1		36	2	1355,71	-0,23
WISKQEYDESGPSIVHRKCF	60	ACTB	yes	21	4	2409,16	-0,51
IIDPGDSDIIRSmPEQTGEK	6156	RPL30		21	3	2217,07	0,33
AIIDPGDSDIIRSmPEQTGEK	6156	RPL30		20	3	2288,11	0,13
VTYVPVTTFKNLQTVNVDEN	6160	RPL31		47	2	2281,18	2,27
QVTQPTVGmNFKTPRGPV	6218	RPS17		40	3	1973,03	-0,42
EVSVNQSLLQPLNVKVD	738196	KRT2		60	2	1882,03	0,86
m = oxidized Methionin							

Eluted from: BLS-DR2a using panHLA-class II specific antibody (L243)

Sequence	Gene ID	Protein	multiple protein hits	Ion-Score	Charge	MH+ [Da]	ΔM [ppm]
FQTLVMLETVPRSGEV	100290966 3127 3125 3126	HLA-DRB1 HLA-DRB5 HLA-DRB3 HLA-DRB4	yes	47	2	1805,95	1,63
FQTLVMLETVPRSGEVY	100290966 3127 3125 3126	HLA-DRB1 HLA-DRB5 HLA-DRB3 HLA-DRB4	yes	45	2	1969,01	1,76

DGKDYIALNEDLRSW	3107	HLA-C	yes	39	3	1794,86	-0,46
DGKDYLALNEDLRSW	3135	HLA-G	yes	39	3	1794,86	-0,46
DVGEYRAVTELGRPD	3123 3126 3127 100509246	HLA-DRB1 HLA-DRB4 HLA-DRB5 LOC100509246	yes	34	3	1676,83	2,02
TQSYSLQLSNLKMED	114836	SLAMF6		65	2	1756,84	0,95
TQSYSLQLSNLKMEDT	114836	SLAMF6		42	2	1857,89	0,60
FTQSYSLQLSNLKMEDTG	114836	SLAMF6		41	2	2061,98	0,81
IHSLPPEGKLGIMEL	1351	COX8A		23	3	1633,90	-0,48
GIPEEELLRQQQELFAK	1810	DR1		22	3	2028,08	2,81
LPVPAFNVINGGSHAG	2023	ENO1	yes	78	2	1549,81	0,13
FELFPSLSHNLLVD	23549	DNPEP		20	2	1630,85	0,44
DAPMFVMGVNHEKYDN	2597	GAPDH		38	2	1866,82	0,70
DAPmFVMGVNHEKYDN	2597	GAPDH		56	2	1882,81	2,23
DAPMFVMGVNHEKYDNSL	2597	GAPDH		36	2	2066,93	1,57
ISWYDNEFGYSNRVVDL	2597	GAPDH		40	2	2076,97	1,48
DAPMFVMGVNHEKYDNSLK	2597	GAPDH		33	3	2195,03	-0,03
FPTIYFSPANKKLN	2923	PDIA3		23	3	1639,88	-0,67
GFPTIYFSPANKKLNPK	2923	PDIA3		28	3	1922,06	3,23
FPTIYFSPANKKLNPK	2923	PDIA3		31	2	1865,04	2,42
ATRLKGIVPLAKVD	2923	PDIA3		30	3	1480,92	-1,39
AHSEFLKAASNLRDNYR	2923	PDIA3		39	4	1992,00	-0,86
INKYVTILHLPKKGDD	3001	GZMA		26	4	1854,05	-0,70
GPPVSELITKAVAASKERSG	3008	HIST1H1E		30	4	1997,10	-1,66
YDMNAANVGWNNSTFA	4282	MIF		52	2	1774,75	1,12
YDmNAANVGWNNSTFA	4282	MIF		46	2	1790,75	1,09

IVNTNVPRASVPDGFLSEL	4282	MIF		39	2	2028,08	1,43
DVSPSDPKIQEVYIP	5337	PLD1		25	2	1686,86	1,16
SYLDPPDLPSNSNDDLLSL	57178	ZMIZ1		20	2	2074,99	2,18
LSYLDPPDLPSNSNDDLLSL	57178	ZMIZ1		40	2	2188,07	2,03
VDIFQVVKALRKA	5788	PTPRC		32	3	1486,91	-1,11
VVDIFQVVKALRKARPG	5788	PTPRC		21	3	1896,15	-0,22
QVTQPTVGMNFKTPRGPV	6218	RPS17		46	3	1957,03	0,85
YVQSmDVAAFNKI	644820	EEF1B4	yes	34	2	1501,74	0,27
FEDYVQSMDVAAFNKI	1933	EEF1B2	yes	66	2	1876,88	1,39
PAEDWAGLDEDEDAHVWEDN	7979	SHFM1		68	2	2312,93	3,11
WQVKSGTIFDNF	811	CALR		44	2	1441,71	0,34
MIDVTKSYYQKFLPLTQV	84516	DCTN5		23	3	2174,16	-0,29
TPVYLGATAGMRLL	953	ENTPD1		78	2	1462,81	1,03
LMQALPMGALPQ	972	CD74		30	2	1269,67	0,02
KPPKPVSKmRMATPLLMQA	972	CD74	yes	27	3	2140,18	-0,57
TPLLmQALPMGALP	972	CD74	yes	22	2	1468,79	0,26
ATPLLMQALPMGALP	972	CD74	yes	22	2	1523,83	-0,45
ATPLLmQALPMGALP	972	CD74	yes	28	2	1539,83	-1,42
ATPLLMQALPmGALP	972	CD74	yes	29	2	1539,83	0,87
TPLLMQALPmGALPQ	972	CD74	yes	40	2	1596,85	1,86
TPLLmQALPmGALPQ	972	CD74	yes	23	2	1612,85	1,13
LPKPPKPVSKMRMATPLLMQAL	972	CD74	yes	24	3	2447,41	0,70
ATPLLMQALPMGALPQ	972	CD74	yes	33	2	1651,89	1,22
MATPLLMQALPMGALP	972	CD74	yes	43	2	1654,88	1,52
ATPLLMQALPmGALPQ	972	CD74	yes	28	2	1667,89	1,11
mATPLLMQALPMGALP	972	CD74	yes	61	2	1670,87	0,53
MATPLLMQALPmGALP	972	CD74	yes	35	2	1670,87	0,53

MATPLLmQALPMGALP	972	CD74	yes	38	2	1670,87	0,90
mATPLLmQALPMGALP	972	CD74	yes	41	2	1686,86	-0,30
mATPLLMQALPmGALP	972	CD74	yes	50	2	1686,86	0,72
mATPLLMQALPMGALPQ	972	CD74	yes	54	2	1798,93	1,23
ATPLLMQALPMGALPQGP	972	CD74	yes	41	2	1805,97	1,98
mATPLLmQALPMGALPQ	972	CD74	yes	51	2	1814,92	1,68
MATPLLmQALPmGALPQ	972	CD74	yes	47	2	1814,92	1,68
mATPLLmQALPmGALPQ	972	CD74	yes	49	2	1830,92	1,38
ATPLLmQALPmGALPQGP	972	CD74	yes	36	2	1837,96	0,77
KPPKPVSKMRMATPLLMQA	972	CD74	yes	45	2	2124,19	1,40
LPKPPKPVSKMRMATPLLmQ	972	CD74		26	2	2279,28	1,06
KPGQAPRLLIYGASSRATG	3514	immunoglobulin kappa light chain		45	3	1943,08	-1,30

Eluted from: BLS-DR2b using HLA-DR2b-specific antibody (H0427A)

Sequence	Gene ID	Protein	Multiple Protein hits	IonScore	Charge	MH+ [Da]	ΔM [ppm]
DVELDDLGKDEL	10130	PDIA6		40	2	1360,65	1,23
IDLSDVELDDLGKDEL	10130	PDIA6		71	2	1788,88	1,92
IGLAKDDQLKVHGF	10209	EIF1		42	3	1540,85	-1,10
VEIGLAKDDQLKVHGF	10209	EIF1		31	3	1768,95	-2,44
AAmLDTVVFK	10213	PSMD14		46	2	1110,59	-0,95
LGISNLSQVRASN	10592	SMC2		31	2	1358,74	-0,10
FYVGEIIGKRGIIGYDV	10632	ATP5L		27	3	1899,04	-1,27
FGSAFATPFLVVRHQLLKT	1350	COX7C		43	2	2132,20	1,09
ATPFLVVRHQLLKT	1350	COX7C		27	2	1622,97	0,06

FGSAFATPFLVVRHQLLKT	1350	COX7C		21	4	2132,20	0,32
YFGSAFATPFLVVRHQLLKT	1350	COX7C		26	3	2295,27	0,88
IHSLPPEGKLGImEL	1351	COX8A		48	3	1649,89	0,57
IHSLPPEGKLGIMEL	1351	COX8A		27	3	1633,90	-0,71
IHSLPPEGKLGImEL	1351	COX8A		32	3	1649,89	-1,09
AVmGFSGFGSTKKSY	153527	ZMAT2		21	3	1582,76	-0,49
GYLPNQLFRTF	1653	DDX1		37	2	1355,71	-0,65
LHLGYLPNQLFRTF	1653	DDX1		35	2	1718,94	0,38
LHLGYLPNQL	1653	DDX1		31	2	1167,65	-0,87
FSNKITPIQSKEAY	205	AK4	yes	25	3	1625,85	-0,96
PSLSHNLLVD	23549	DNPEP		29	2	1094,58	-0,17
ISWYDNEFGYSNRVVDL	2597	GAPDH		42	2	2076,97	1,25
VGAHAGEYGAEALERm	3039	HBA1		34	3	1676,77	-0,97
GKVGAHAGEYGAEALERm	3039	HBA1	yes	29	3	1861,88	-1,82
FRDGDILGKYVD	3336	HSPE1		29	2	1397,71	0,41
IVNTNVPRASVPDGFLSEL	4282	MIF		45	2	2028,08	0,59
NAANVGWNNSTFA	4282	MIF		45	2	1365,62	-0,13
mVGPIEEAVAKADKLAEEHSS	506	ATP5B		26	4	2227,09	0,32
VPPVQVSPLIKLGRYSAL	521	ATP5I		42	3	1937,16	-1,56
KFEDPKFEVIEKPQA	522	ATP5J		43	3	1804,95	0,08
PTFKFEDPKFEVIEKPQA	522	ATP5J		21	4	2150,12	-0,71
DIAVDGEPLGRVSFEL	5478	PPIA		66	2	1716,88	0,51
FVILRKNPNYDL	5708	PSMD2		24	2	1491,83	0,54
LVSNLNPERVTPQSLFIL	5725	PTBP1		21	2	2040,15	-0,14
ISKQEYDESGPSIVHRKCF	60	ACTB	yes	67	2	2223,08	-1,67
WISKQEYDESGPSIVHRKCF	60	ACTB	yes	67	2	2409,17	1,58
AIIDPGDSDIIRSmPEQTGEK	6156	RPL30		45	3	2288,11	0,05

IIDPGDSDIIRSMPEQTGEK	6156	RPL30		27	3	2201,08	0,38
YVPVTTFKNLQTVNVDEN	6160	RPL31		56	2	2081,06	0,68
VTYVPVTTFKNLQTVNVDEN	6160	RPL31		42	2	2281,18	2,38
mRYVASYLLAALGGN	6181	RPLP2		26	2	1614,83	1,63
FSLPIKESEIIDF	6187	RPS2	yes	38	2	1537,82	0,34
QVTQPTVGMNFKTPRGPV	6218	RPS17		67	2	1957,03	1,01
YIRGVEEEEEDGEmRE	6636	SNRPF		37	3	1985,84	0,37
GDVKPVVSSTPLVDFL	6881	TAF10		37	2	1672,92	1,31
FYGQTLGQAQAHSQEQ	8939	FUBP3		37	2	1792,83	1,32
IRPEIHENYRING	9550	ATP6V1G1		22	3	1610,84	-0,38
FVCDIRPEIHENYRING	9550	ATP6V1G1		33	3	2075,01	-1,39

Eluted from: BLS-DR2b using panHLA-class II-specific antibody (L243)

Sequence	Gene ID	Protein	multiple protein hits	IonScore	Charge	MH+ [Da]	ΔM [ppm]
LEEFGRFASF	3122	HLA-DRA		49	2	1202,58	0,06
LEEFGRFASFEAQGA	3122	HLA-DRA		46	2	1658,78	0,47
LEEFGRFASFEAQGAL	3122	HLA-DRA		40	2	1771,86	-0,23
FGRFASFEAQG	3122	HLA-DRA		33	2	1216,57	-1,09
FGRFASFEAQGAL	3122	HLA-DRA		47	2	1400,69	-0,84
LEEFGRFASFEAQG	3122	HLA-DRA		44	2	1587,75	1,88
ATPFLVVRHQLLKT	1350	COX7C		22	3	1622,97	-0,78
IHSLPPEGKLGIMEL	1351	COX8A		20	3	1633,89	-2,22
GYLPNQLFRTF	1653	DDX1		46	2	1355,71	-0,41
QADLSSFKSQELNER	2208	FCER2		87	2	1751,86	1,22
LQADLSSFKSQELNERN	2208	FCER2		55	3	1978,98	-0,25

QADLSSFKSQELNERN	2208	FCER2		34	3	1865,90	-1,85
QADLSSFKSQELNER	2208	FCER2		29	3	1751,86	0,06
LSSFKSQELNER	2208	FCER2		26	3	1437,73	-0,03
ATRLKGIVPLAKVD	2923	PDIA3		32	3	1480,92	-0,83
GFPTIYFSPANKKLNPK	2923	PDIA3		27	3	1922,05	-0,77
SLDDLQPWHSFGADS	351	APP		47	2	1674,74	1,04
LDDLQPWHSFGADS	351	APP		45	2	1587,71	0,75
SLDDLQPWHSFGADSVPA	351	APP		32	2	1941,90	1,44
DVSPSDPKIQEVYIP	5337	PLD1		26	2	1686,86	2,07
AIIDPGDSDIIRSMPEQTGEK	6156	RPL30		25	3	2272,11	0,17
VTYVPVTTFKNLQTVNVDEN	6160	RPL31		47	2	2281,18	1,95
QVTQPTVGMNFKTPRGPV	6218	RPS17		51	3	1957,03	-0,93
YVQSMDVAAFNKI	644820	EEF1B4		75	2	1485,74	0,52
WISKQEYDESGPSIVHRKCF	71	ACTG1	yes	49	3	2409,17	0,91
ISKQEYDESGPSIVHRKCF	71	ACTG1	yes	41	3	2223,09	-0,23
QQMWISKQEYDESGPSIVHRKCF	71	ACTG1	yes	22	4	2796,32	-1,03
WQVKSGTIFDNF	811	CALR		31	2	1441,71	-0,25
KPPKPVSKmRMATPLLMQA	972	CD74	yes	28	3	2140,18	0,17
MATPLLMQALPMGALP	972	CD74	yes	26	2	1654,88	1,59
ATPLLMQALPMGALPQGP	972	CD74	yes	23	2	1805,97	0,43
KPPKPVSKMRmATPLLMQA	972	CD74	yes	22	3	2140,18	0,00
KPPKPVSKMRMATPLLMQAL	972	CD74	yes	20	3	2237,27	-0,02
MATPLLMQALPmGALPQ	972	CD74	yes	37	2	1798,93	0,81

Suppl. Table 3: Biological pathway gene ontology annotation analysis of DR15-eluted peptides

Gene Ontology Biological Pathway Term	Count	PValue	Gene ID (Gene)	
antigen processing and presentation	6	1,2E+11	811 (CALR) 3135 (HLA-G) 972 (CD74)	3127 (HLA-DRB) 3107 (HLA-C) 3122 (HLA-DRA)
generation of precursor metabolites and energy	8	1,2E+11	522 (ATP5J) 521 (ATP5I) 1350 (COX7C) 10632 (ATP5L)	506 (ATP5B) 1351 (COX8A) 2597 (GAPDH) 2023 (ENO1)
translational elongation	6	3,1E+10	6181 (RPLP2) 6160 (RPL31) 6187 (RPS2)	1933 (EEF1B2) 6218 (RPS17) 6156 (RPL30)
regulation of cellular protein metabolic process	7	0,00699	811 (CALR) 5708 (PSMD2) 10213 (PSMD14) 351 (APP)	1653 (DDX1) 10209 (EIF1) 5788 (PTPRC)
immune response	8	0,01157	4282 (MIF) 3135 (HLA-G) 3107 (HLA-C) 5788 (PTPRC)	3127 (HLA-DRB) 3001 (GZMA) 972 (CD74) 3122 (HLA-DRA)
cellular homeostasis	6	0,02609	811 (CALR) 10130 (PDIA6) 5788 (PTPRC)	506 (ATP5B) 2923 (PDIA4) 351 (APP)

macromolecular complex assembly	7	0,03201	4282 (MIF) 1653 (DDX1) 972 (CD74) 3008 (HIST1H1E)	811 (CALR) 6636 (SNRPF) 6881 (TAF10)
positive regulation of catalytic activity	6	0,03916	5708 (PSMD2) 10213 (PSMD14) 3336 (HSPE1)	2208 (FCER2) 972 (CD74) 5788 (PTPRC)
macromolecular complex subunit organization	7	0,04203	4282 (MIF) 1653 (DDX1) 972 (CD74) 3008 (HIST1H1E)	811 (CALR) 6636 (SNRPF) 6881 (TAF10)
cation transport	6	0,04884	522 (ATP5J) 521 (ATP5I) 10632 (ATP5L)	506 (ATP5B) 9550 (ATP6V1G1) 5788 (PTPRC)

Suppl. Table 4: HLA-DR15 haplotype-derived self-peptides

Pool Number	Peptide Number	Origin	Sequence	Remark
1	1	DRB1*15:01	MVCLK LPGGS CMTAL	
1	2	DRB1*15:01	CMTAL TVTLM VLSSP	
1	3	DRB1*15:01	VLSSP LALSG DTRPR	
1	4	DRB1*15:01	DTRPR FLWQP KRECH	
1	5	DRB1*15:01	KRECH FFNGT ERVRF	
2	6	DRB1*15:01	ERVRF LDRYF YNQEE	
2	7	DRB1*15:01	YNQEE SVRFD SDVGE	
2	8	DRB1*15:01	SDVGE FRAVT ELGRP	
2	9	DRβ	ELGRP DAEYW NSQKD	shared
2	10	DRB1*15:01	NSQKD ILEQA RAAVD	
3	11	DRB1*15:01	RAAVD TYCRH NYGVV	
3	12	DRB1*15:01	NYGVV ESFTV QRRVQ	
3	13	DRB1*15:01	QRRVQ PKVTV YPSKT	
3	14	DRB1*15:01	YPSKT QPLQH HNLLV	
3	15	DRB1*15:01	HNLLV CSVSG FYPGS	
4	16	DRB1*15:01	FYPGS IEVRW FLNGQ	
4	17	DRB1*15:01	FLNGQ EEKAG MVSTG	
4	18	DRB1*15:01	MVSTG LIQNG DWTFQ	
4	19	DRβ	DWTFQ TLVML ETVPR	shared
4	20	DRβ	ETVPR SGEVY TCQVE	shared
5	21	DRβ	TCQVE HPSVT SPLTV	shared
5	22	DRB1*15:01	SPLTV EWRAR SESAQ	
5	23	DRβ	SESAQ SKMLS GVGGF	shared
5	24	DRβ	GVGGF VLGLL FLGAG	shared

5	**25**	DRB1*15:01	FLGAG LFIYF RNQKG
5	**26**	DRB1*15:01	RNQKG HSGLQ PTGFLS
6	**27**	DRB5*01:01	MVCLK LPGGS YMAKL
6	**28**	DRB5*01:01	YMAKL TVTLM VLSSP
6	**29**	DRB5*01:01	VLSSP LALAG DTRPR
6	**30**	DRB5*01:01	DTRPR FLQQD KYECH
6	**31**	DRB5*01:01	KYECH FFNGT ERVRF
7	**32**	DRB5*01:01	ERVRF LHRDI YNQEE
7	**33**	DRB5*01:01	YNQEE DLRFD SDVGE
7	**34**	DRB5*01:01	SDVGE YRAVT ELGRP
7	**35**	DRB5*01:01	NSQKD FLEDR RAAVD
7	**36**	DRB5*01:01	RAAVD TYCRH NYGVG
8	**37**	DRB5*01:01	NYGVG ESFTV QRRVE
8	**38**	DRB5*01:01	QRRVE PKVTV YPART
8	**39**	DRB5*01:01	YPART QTLQH HNLLV
8	**40**	DRB5*01:01	HNLLV CSVNG FYPGS
8	**41**	DRB5*01:01	FYPGS IEVRW FRNSQ
9	**42**	DRB5*01:01	FRNSQ EEKAG VVSTG
9	**43**	DRB5*01:01	VVSTG LIQNG DWTFQ
9	**44**	DRB5*01:01	SPLTV EWRAQ SESAQ
9	**45**	DRB5*01:01	FLGAG LFIYF KNQKG
9	**46**	DRB5*01:01	KNQKG HSGLH PTGLVS
10	**47**	DRA*01:01	MAISG VPVLG FFIIA
10	**48**	DRA*01:01	FFIIA VLMSA QESWA
10	**49**	DRA*01:01	QESWA IKEEH VIIQA
10	**50**	DRA*01:01	VIIQA EFYLN PDQSG
10	**51**	DRA*01:01	PDQSG EFMFD FDGDE

11	**52**	DRA*01:01	FDGDE IFHVD MAKKE	
11	**53**	DRA*01:01	MAKKE TVWRL EEFGR	
11	**54**	DRA*01:01	EEFGR FASFE AQGAL	
11	**55**	DRA*01:01	AQGAL ANIAV DKANL	
11	**56**	DRA*01:01	DKANL EIMTK RSNYT	
12	**57**	DRA*01:01	RSNYT PITNV PPEVT	
12	**58**	DRA*01:01	PPEVT VLTNS PVELR	
12	**59**	DRA*01:01	PVELR EPNVL ICFID	
12	**60**	DRA*01:01	ICFID KFTPP VVNVT	
12	**61**	DRA*01:01	VVNVT WLRNG KPVTT	
13	**62**	DRA*01:01	KPVTT GVSET VFLPR	
13	**63**	DRA*01:01	VFLPR EDHLF RKFHY	
13	**64**	DRA*01:01	RKFHY LPFLP STEDV	
13	**65**	DRA*01:01	STEDV YDCRV EHWGL	
13	**66**	DRA*01:01	EHWGL DEPLL KHWEF	
14	**67**	DRA*01:01	KHWEF DAPSP LPETT	
14	**68**	DRA*01:01	LPETT ENVVC ALGLT	
14	**69**	DRA*01:01	ALGLT VGLVG IIIGT	
14	**70**	DRA*01:01	IIIGT IFIIK GVRKS	
14	**71**	DRA*01:01	GVRKS NAAER RGPL	
15	**72**	DQA1*01:02	MILNK ALLLG ALALT	
15	**73**	DQA1*01:02	ALALT TVMSP CGGED	
15	**74**	DQA1*01:02	CGGED IVADH VASCG	
15	**75**	DQA1*01:02	VASCG VNLYQ FYGPS	
15	**76**	DQA1*01:02	FYGPS GQYTH EFDGD	
16	**77**	DQA1*01:02	EFDGD EQFYV DLERK	
16	**78**	DQA1*01:02	DLERK ETAWR WPEFS	

16	**79**	DQA1*01:02	WPEFS KFGGF DPQGA	
16	**80**	DQA1*01:02	DPQGA LRNMA VAKHN	
16	**81**	DQA1*01:02	VAKHN LNIMI KRYNS	
17	**82**	DQA1*01:02	KRYNS TAATN EVPEV	
17	**83**	DQA1*01:02	EVPEV TVFSK SPVTL	
17	**84**	DQA1*01:02	SPVTL GQPNT LICLV	
17	**85**	DQA1*01:02	LICLV DNIFP PVVNI	
17	**86**	DQA1*01:02	PVVNI TWLSN GQSVT	
18	**87**	DQA1*01:02	GQSVT EGVSE TSFLS	
18	**88**	DQA1*01:02	TSFLS KSDHS FFKIS	
18	**89**	DQA1*01:02	FFKIS YLTFL PSADE	
18	**90**	DQA1*01:02	PSADE IYDCK VEHWG	
18	**91**	DQA1*01:02	VEHWG LDQPL LKHWE	
19	**92**	DQA1*01:02	LKHWE PEIPA PMSEL	
19	**93**	DQA1*01:02	PMSEL TETVV CALGL	
19	**94**	DQA1*01:02	CALGL SVGLM GIVVG	
19	**95**	DQA1*01:02	GIVVG TVFII QGLRS	
19	**96**	DQA1*01:02	QGLRS VGASR HQGPL	
20	**97**	DQB1*06:02	MSWKK ALRIP GDLRV	
20	**98**	DQB1*06:02	GDLRV ATVTL MLAML	
20	**99**	DQB1*06:02	MLAML SSLLA EGRDS	
20	**100**	DQB1*06:02	EGRDS PEDFV FQFKG	
20	**101**	DQB1*06:02	FQFKG MCYFT NGTER	
21	**102**	DQB1*06:02	NGTER VRLVT RYIYN	
21	**103**	DQB1*06:02	RYIYN REEYA RFDSD	
21	**104**	DQB1*06:02	RFDSD VGVYR AVTPQ	
21	**105**	DQB1*06:02	AVTPQ GRPDA EYWNS	

21	**106**	DQB1*06:02	EYWNS QKEVL EGTRA	
22	**107**	DQB1*06:02	EGTRA ELDTV CRHNY	
22	**108**	DQB1*06:02	CRHNY EVAFR GILQR	
22	**109**	DQB1*06:02	GILQR RVEPT VTISP	
22	**110**	DQB1*06:02	VTISP SRTEA LNHHN	
22	**111**	DQB1*06:02	LNHHN LLVCS VTDFY	
23	**112**	DQB1*06:02	VTDFY PGQIK VRWFR	
23	**113**	DQB1*06:02	VRWFR NDQEE TAGVV	
23	**114**	DQB1*06:02	TAGVV STPLI RNGDW	
23	**115**	DQB1*06:02	RNGDW TFQIL VMLEM	
23	**116**	DQB1*06:02	VMLEM TPQRG DVYTC	
24	**117**	DQB1*06:02	DVYTC HVEHP SLQSP	
24	**118**	DQB1*06:02	SLQSP ITVEW RAQSE	
24	**119**	DQB1*06:02	RAQSE SAQSK MLSGV	
24	**120**	DQB1*06:02	MLSGV GGFVL GLIFL	
24	**121**	DQB1*06:02	GLIFL GLGLI IRQRS	
24	**122**	DQB1*06:02	IRQRS QKGLL H	